Passive stretch regulates skeletal muscle glucose uptake independent of nitric oxide synthase

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Abstract

Skeletal muscle contraction increases glucose uptake via an insulin-independent mechanism. Signaling pathways arising from mechanical strain are activated during muscle contractions, and mechanical strain in the form of passive stretching stimulates glucose uptake. However, the exact mechanisms regulating stretch-stimulated glucose uptake are not known. Since nitric oxide synthase (NOS) has been implicated in the regulation of glucose uptake during ex vivo and in situ muscle contractions and during exercise, and NO is increased with stretch, we examined whether the increase in muscle glucose uptake during stretching involves NOS. We passively stretched isolated EDL muscles (15 min at ~100-130 mN) from control mice and mice lacking either neuronal NOSμ (nNOSμ) or endothelial NOS (eNOS) isoforms, as well as used pharmacological inhibitors of NOS. Stretch significantly increased muscle glucose uptake approximately 2-fold ($P < 0.05$), and this was unaffected by the presence of the NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA; 100 μM) or N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM). Similarly, stretch-stimulated glucose uptake was not attenuated by deletion of either eNOS or nNOSμ isoforms. Furthermore, stretching failed to increase skeletal muscle NOS enzymatic activity above resting levels. These data clearly demonstrate that stretch-stimulated skeletal muscle glucose uptake is not dependent on NOS.

Key words: nitric oxide synthase, glucose uptake, stretch, skeletal muscle

New & Noteworthy

Passive stretching is known to activate muscle glucose uptake through mechanisms that partially overlap with contraction. We report that genetic knockout of eNOS or nNOS or pharmacological NOS inhibition does not affect stretch-stimulated glucose uptake. Passive stretch failed to increase NOS activity above resting levels. This information is important for the study of signaling pathways that regulate stretch-stimulated glucose uptake and indicate that NOS should be excluded as a potential signaling factor in this regard.

INTRODUCTION

Exercise and ex vivo and in situ muscle contractions potently stimulate the uptake of glucose into skeletal muscle via a signaling pathway that is, at least proximally, independent of the canonical insulin signaling pathway (35). Signaling proteins that mediate glucose uptake during exercise present as an attractive therapeutic target for the treatment of Type 2 diabetes since glucose uptake and GLUT-4 translocation during contraction and exercise are mostly normal in insulin resistant muscle (24, 29, 53). However, the exact mechanisms involved remain to be fully clarified.

The transduction of mechanical stimuli into biochemical signals has long been known to regulate biological processes in skeletal muscle (9, 16, 50). Several studies have shown that mechanical loading applied to isolated rodent muscles in the form of passive stretching increases muscle glucose uptake (5, 18, 20, 23, 45), presumably via stimulating GLUT4 translocation (45). It is likely that a mechanical signaling component is essential to fully activate the glucose transport machinery during contractions, as the prevention of tension development during electrically-induced skeletal muscle contractions attenuates the increase in glucose uptake (2, 18, 23, 45). While muscle contractions have been shown to induce metabolic disturbances and activation of AMP-activated protein kinase (AMPK), this pathway is not activated by stretch (5, 23, 45). On the other hand, passive stretching activates the cytoskeletal regulator Rac1, and Rac1 inhibition has been shown to attenuate stretch-stimulated glucose uptake (44, 45). However, Rac1 inhibition does not affect the increase in glucose uptake during electrical stimulations when tension development is prevented (45). This indicates that during muscle contraction mechanical stimuli activates a

distinct signaling pathway that contributes to glucose uptake. The exact signaling mechanisms involved in this pathway are not known.

Nitric oxide synthase (NOS) activity and nitric oxide (NO) production is increased during electrical stimulations in muscle cells (34, 42), muscle contractions or exercise in rodents (14, 15, 31, 32, 36, 38), and exercise in humans (28). Several studies have demonstrated that pharmacological inhibition of NOS attenuates the increase in skeletal muscle glucose uptake during contractile activity (1, 3, 14, 24, 31, 32, 37, 38), although this is not a universal finding (7, 10, 12, 13, 39). Neuronal NOSμ (nNOSμ) is considered the predominant source of NO in contracting skeletal muscle (14, 26) and is largely targeted to the mechanosensing dystrophin-glycoprotein complex (DGC) at the sarcolemma (4). Acute passive stretch of both muscle cells and mature muscle has also been reported to increase NO production (48, 54, 55), and there is evidence that NOS is involved in the transduction of mechanical signal pathways regulating the expression of cytoskeletal proteins (49). Given that NO contributes to the regulation of glucose uptake during muscle contractions, NO production is increased by stretch and NOS can participate in mechanical signaling, it is tempting to speculate that mechanical-stress (stretch) regulates glucose uptake via a NOS-dependent mechanism. However, to the best of our knowledge, no previous study has investigated the role of NO in the regulation of this pathway.

Therefore, the aim of this study was to determine whether acute passive stretch regulates glucose uptake via a NOS-dependent pathway. To determine this, we used two genetically modified mouse models lacking either eNOS or nNOSμ and two pharmacological NOS inhibitors which target all NOS isoforms. We hypothesized

that stretch-stimulated glucose uptake in mouse EDL muscle would be attenuated by NOS inhibition and/or genetic deletion of nNOSμ.

Materials and Methods

Animals

All animal experimentation was conducted at the Institute of Sport, Exercise & Active Living (ISEAL), Victoria University, Melbourne with the prior approval of the Victoria University Animal Ethics Committee. Animal experimentation adhered to the Australian Code of Practice for the use and care of animals for scientific purposes as described by the National Health and Medical Research Council (NHMRC) of Australia. Thirteen- to sixteen-week-old C57BL/6, eNOS knockout ($eNOS^{-/-}$), and nNOSμ knockout ($nNOS\mu^{-/-}$) mice were involved in this study. Six male mice lacking eNOS ($eNOS^{-/-}$) (Monash Animal Services, Melbourne, Australia) and eight male C57BL/6 mice (ARC, Perth, Australia) aged 14–16 weeks were used to examine the role of eNOS. The $eNOS^{-/-}$ group was generated by using $eNOS^{-/-}$ breeding pairs and therefore wildtype littermates ($eNOS^{+/+}$) were not produced. Since these mice were generated on a C57BL/6 background we chose to use C57BL/6 mice as controls. Seven $nNOS\mu^{-/-}$ mice and six wildtype littermates ($nNOS^{+/+}$) (male and female) aged 13–15 weeks were used to examine the role of nNOSμ. $nNOS\mu^{-/-}$ (B6, 129-$NOS1^{tm1plh}$) mice were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA, stock no. 002633) (17) and backcrossed onto a C57BL/6 background for at least six generations to obtain a colony of $nNOS^{-/-}$ and wild type littermate controls. Male C57BL/6 mice aged 13–15 weeks (ARC, Perth, Australia) were used for NOS inhibitor and NOS activity experiments. Mice were housed in standard cages and

maintained at 21°C on a 12-hour dark/light cycle with access to water and standard rodent chow ad libitum. Mice were not fasted prior to sacrifice.

Muscle incubations

NOS inhibition in mice has previously been shown to attenuate the increase in contraction-stimulated glucose uptake in extensor digitorum longus (EDL) muscles, but not soleus muscles (32). In addition, electrical stimulations have been shown to elevate levels of the NO downstream intermediate cGMP in EDL but not soleus muscles (26). Therefore, only EDL muscles were examined in the present study. EDL muscles were excised from anaesthetized mice (sodium pentobarbitone 70 mg/kg IP) and suspended at resting length (~2–4 mN) (45) in organ baths (MultiMyograph System; Danish Myotechnology, Aarhus, Denmark). All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Muscles were pre-incubated for 30 min in Krebs-Ringer-Henseleit buffer consisting of (mM): NaCl 118.5, $NaHCO_3$ 24.7, KCl 4.74, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.5, (pH 7.4) supplemented with 0.01% BSA (Cat. # A2153), 8 mM mannitol and 2 mM sodium pyruvate. Incubation media was maintained at 30°C and continuously oxygenated with gas containing 95% O_2 and 5% CO_2. Following the 30 min pre-incubation period, muscles either remained at rest or were stretched to a tension of 100–130 mN for 15 minutes (44, 45). When the effects of the NOS inhibitors N^G-monomethyl-L-arginine (L-NMMA, 100 μM) (12, 40), and N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) (40) were examined, these inhibitors were present during the entire 45 min incubation time. L-NMMA at this concentration has previously been shown to attenuate the increase in NOS activity by ~90% (12, 31, 40) and contraction-stimulated glucose uptake during contraction ex vivo in mouse EDL

by ~20-50% (14, 31, 32). L-NAME has previously been shown to exert a similar dose-dependent inhibitory effect as L-NMMA on NOS activity in skeletal muscle (40). Immediately following the 45-min experimental period, muscles were quickly removed from the organ baths, washed in ice-cold Kreb's buffer, blotted dry on filter paper, snap frozen in liquid nitrogen, and stored at -80°C for future analysis.

Muscle processing

To generate lysates for immunoblotting and NOS activity measurement, whole frozen EDL muscles were homogenized in ice-cold buffer [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μl/ml protease inhibitor mixture, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate] by steel beads for 2 x 30 s 30 Hz (TissueLyser, Qiagen, Valencia, CA), followed by end-over-end rotation for 30 min at 4°C. Homogenates were centrifuged at 10,000 g for 20 min at 4°C, and the supernatant collected for NOS activity measurement. For immunoblotting, an aliquot was collected prior to the centrifugation step and diluted in sample buffer (0.125 M Tris-HCl [pH 6.8], 4% SDS, 10% glycerol, 10 mM EGTA, 0.1 M DTT and 0.01% bromophenol blue) and heated at 95°C for 10 min before being subjected to SDS-PAGE. Protein concentration was determined by the Red660 protein assay kit (G Biosciences, St Louis, MO).

Immunoblotting

Total protein (5 μg) was separated by SDS-PAGE using stain-free gels (Bio-Rad, Hercules, CA) and semi-dry transferred (TransBlot Turbo system, Bio-Rad) to PVDF membranes. Prior to transfer, a stain-free image of the gel was collected to quantify

total protein loading. Stain-free gel analysis indicated that no differences in protein loading were observed. Membranes were blocked for one hour at room temperature (5% skim milk in TBST), before being probed overnight at 4°C with the following primary antibodies: p-PAK1/2$^{Thr423/Thr402}$ (1:500), p-P38 MAPK$^{Thr180/Tyr182}$ (1:1000), p-AMPK^{Thr172} (1:1000), and p-CaMKII^{Thr286} (1:1000) (Cell Signaling Technology). The following day, membranes were incubated with HRP-secondary antibody for 1 hour at room temperature. Protein bands were visualized using Bio-Rad ChemiDoc imaging system and enhanced chemiluminescence substrate (SuperSignal West Femto, Pierce, MA), and quantified using ImageLab software (Bio-Rad). Analysis of protein bands were normalized to stain-free quantification of protein loading.

NOS activity and glucose uptake measurements

NOS activity was determined on muscle lysates in duplicate by measuring the conversion of L-[^{14}C] arginine to L-[^{14}C] citrulline (14, 27). Muscle glucose uptake was calculated during the final 10 minutes of stretch or basal conditions by exchanging the incubation buffer with buffer containing 1 mM 2-deoxy-D-[1,2-^{3}H] glucose (0.128 µCi/mL) and 8 mM D-[^{14}C] mannitol (0.083 µCi/mL) (Perkin Elmer, Boston, MA) as described previously (14).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6.0 software. Glucose uptake was analyzed using one (treatment)- and two (treatment and genotype)-factor ANOVA. Fisher's least significance difference test was performed if the ANOVA revealed a significant difference. Students t-test was used to compare morphological characteristics between each

genotype and its relevant control, NOS activity and protein phosphorylation. The significance level was set at $P < 0.05$.

RESULTS

Morphology characteristics of NOS knockout mice

Body mass was not different between C57BL/6 control mice and $eNOS^{-/-}$ mice (28.1 ± 0.8 vs. 27.4 ± 1.3 g; P = 0.65; n = 6-8) or between $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ mice (24.1 ± 1.1 vs. 22.4 ± 0.6 g; P = 0.17 n = 6-7). EDL muscle mass was significantly lower in $nNOS\mu^{-/-}$ compared with $nNOS\mu^{+/+}$ mice (7.1 ± 0.2 vs. 8.6 ± 0.3 mg; $P < 0.001$; n = 12-14), whereas EDL mass was similar between C57BL/6 control mice and $eNOS^{-/-}$ mice (10.5 ± 0.4 vs. 10.1 ± 0.4 mg; P = 0.49 n = 11-15).

Stretch-stimulated glucose uptake

To investigate the involvement of NOS in the regulation of glucose uptake in response to mechanical loading, we examined the effects on stretch-stimulated glucose uptake in EDL muscle of 1) pharmacological NOS inhibition, and 2) deletion of either eNOS or nNOSμ. In muscles from C57BL/6 mice, passive stretch significantly increased glucose uptake approximately 2-fold compared with basal levels ($P < 0.001$) (Figure 1). Stretch-stimulated glucose uptake was unaffected by the presence of either of the NOS inhibitors L-NMMA or L-NAME (Figure 1). In muscles from $eNOS^{-/-}$ mice, stretch increased glucose uptake approximately 2-fold from basal levels ($P < 0.001$) with a similar increase observed in C57BL/6 control mice (Figure 2A). Stretch also increased glucose uptake approximately 2-fold in muscles from $nNOS\mu^{-/-}$ and $nNOS\mu^{+/+}$ mice ($P < 0.001$). There was a main effect for $nNOS\mu^{-/-}$ indicating that muscles from these mice had elevated basal and stretch-

activated glucose uptake compared with controls (P = 0.02) (Figure 2B). The delta stretch-stimulated glucose uptake (the difference between basal and stretch values) was similar between nNOSμ$^{-/-}$ and nNOSμ$^{+/+}$. These results indicate that NOS is not necessary for normal increases in stretch-stimulated muscle glucose uptake.

Effect of stretch on NOS activity and protein signaling

Stretch did not increase skeletal muscle NOS activity above basal levels (measure of contribution from both eNOS and nNOS) in EDL muscles from C57BL/6 mice (Figure 3A). This was consistent with the lack of effect of deletion of nNOSμ or eNOS, and the lack of effect of NOS inhibition on stretch-stimulated skeletal muscle glucose uptake. To confirm that our stretch protocol did actually activate pathways previously shown to be activated by stretch (5, 23, 45), we examined the phosphorylation status of p38 MAPK as well as the activation of the cytoskeletal regulator Rac1 (22) by examining phosphorylation of the Rac1 downstream kinase PAK1/2 (44, 45, 51). Consistent with previous studies, stretch significantly increased the phosphorylation status of PAK1/2$^{Thr423/402}$ and p38 MAPK$^{Thr180/Tyr182}$ (~2-fold) ($P < 0.05$) (Figure 3B and C) (5, 23, 44, 45). Skeletal muscle p-AMPK^{Thr172} did not increase with stretch which was also consistent with previous research (5, 23, 45) (Figure 3D). Likewise, stretch also failed to increase p-CaMKII^{Thr286} (Figure 3E).

DISCUSSION

The major finding of this study was that skeletal muscle stretch-induced increases in glucose uptake are independent of NOS. Given that stretch activated Rac1 (as shown by increased PAK1/2 phosphorylation) but did not activate NOS, it appears that although nNOSμ is part of the dystrophin glycoprotein complex and linked to the

cytoskeleton, stretch induces glucose uptake via the cytoskeleton independently of nNOSμ. In addition, eNOS is also not required for this process.

The lack of NOS activation in EDL muscles following stretch is in contrast with other muscle models whereby stretch increased NO production (43, 47, 48, 54, 55). A key difference is that most of these studies were conducted in cultured muscle cells where a much longer stretching/loading protocol (1 – 48 hours) was applied. Therefore, the increased NO production reported in these chronic stretch studies may have reflected an increased NOS protein content (55) rather than activation of the existing NOS. To our knowledge, only one previous study examined whether acute stretching was sufficient to stimulate NO production in mature intact muscle (48). Tidball and colleagues (48) reported a significant increase (~20%) in NO production from isolated rat soleus muscles following a brief stretch (2 min). In the present study, NO production probably did not increase with stretch-stimulation given that NOS activity was not enhanced above resting levels. This inference is in agreement with a previous study where stretched single mature muscle fibers (10 min) loaded with a NO-sensitive fluorescent probe (DAF-FM), which allowed for a more direct NO estimation, did not have an increase in NO production (33). It is possible, however, that static stretching stimulates an initial burst of NOS activity/NO production that diminishes rapidly over time and was therefore not detected at the time of muscle harvest in our study (15 min). Indeed, it has been shown that shear stress applied to endothelial cells resulted in a marked increase in NO production within 5 minutes followed by little additional NO production thereafter (6). In another study, muscle NOS activity was significantly elevated 3 minutes following the induction of increased load applied to plantaris muscles in mice, and despite the continued load,

NOS activity returned to baseline levels within 1 hour (19). That study is difficult to interpret, however, given that tendons of synergist muscles were ablated resulting in "functional overload" of plantaris muscle and the time of overload was defined as 3 minutes after mice started walking post-surgery (19). In our study, it should also be noted that we did not measure the muscle length required to achieve the passive tension of 100-130 mN. It is possible that loss of NOS isoforms, or the presence of NOS inhibitors could have affected the amount of stretch that was required to be applied to the muscle to achieve the desired passive tension. Nevertheless, the lack of increase in NOS activity with stretch fits with the observation that stretch-stimulated glucose uptake was not attenuated by NOS inhibitors or a lack of nNOSμ or eNOS.

The mechanism(s) by which NOS regulates contraction-stimulated glucose uptake remains to be determined. Since there is emerging evidence glucose uptake is largely regulated by distinct metabolic (AMPK)- and mechanical-dependent (Rac1) signaling arms during muscle contraction (23, 46), in this study we examined the potential involvement of NOS in a mechanical-dependent signaling pathway. The lack of involvement of NOS in stretch-stimulated glucose uptake and Rac1 activation indicates that NOS is not involved in the mechanical signaling arm, and by extension the possibility that NOS regulates glucose uptake during contraction via a mechanism coupled with metabolic disturbances. However, this would likely not involve AMPK (46) given we have previously shown that NOS appears to regulate muscle glucose uptake during contraction independently of AMPK (30, 32). Nonetheless, it is important to note that in a recent study (46), although contraction-stimulated glucose uptake was largely attenuated by blockade of both metabolic (AMPK) and mechanical (Rac1) signaling, some increase in glucose uptake with contraction was maintained,

indicating other signaling pathways are likely at play. For example, mTORC2 signaling has been shown to be essential for muscle glucose uptake during exercise in mice independent of AMPK and Rac1 signaling (25). Therefore, further work is required to examine the potential involvement of NOS in other signaling pathways during contraction.

The activation of Rac1 by contraction and stretch is associated with an increase in glucose transport in muscle (44, 45), however, the upstream signaling events involved are largely unknown. Rac1 contains a redox-sensitive motif and it has been reported that activation of Rac1 is favored in the presence of reactive nitrogen species (11). Exposure of C2C12 cells to a NO donor has previously been shown to induce the rapid activation of Rac1 and phosphorylation of its downstream kinase PAK1 (8), indicating that NO is sufficient to stimulate Rac1 activation. Conversely, nNOS and eNOS have been reported to be activated by Rac1 in human aortic endothelial cells (41). These results suggest that NO/NOS could be upstream and/or downstream of Rac1. We measured PAK1 phosphorylation as a surrogate for Rac1 activity and to investigate possible associations between NO and Rac1/PAK1 pathway. Our data indicate that NO is not necessary for Rac1 activation during stretching and vice versa, given that we observed an increase in stretch-stimulated phosphorylation of PAK1 (and presumably Rac1 activity) without changes in NOS activation. Nonetheless, future work is required to clarify whether a NO-Rac1 interaction exists in skeletal muscle under situations where NO bioavailability is increased, such as during muscle contractions (14).

Ca^{2+}/Calmodulin-dependent kinase II (CaMKII) has also been implicated in the regulation of muscle glucose uptake during contractions in mature muscle in situ (52), however, the inability of stretch to enhance the levels of phosphorylated $CaMKII^{Thr286}$ in our study suggest that CaMKII is not coupled with mechanical signaling mechanisms. This contrasts with a study where stretch-stimulated glucose uptake in C2C12 myotubes was blocked by a CaMK inhibitor (21). However, as discussed above, the pathways regulating stretch-stimulated glucose uptake potentially differ between in vitro and ex vivo models.

In conclusion, we have shown that passive stretching does not increase NOS activity in skeletal muscle and stretch-stimulated glucose uptake is not attenuated by either pharmacological inhibition of NOS or by deletion of eNOS or nNOSμ isoforms. Therefore, our results indicate that NOS signaling is not required for stretch-induced increases in skeletal muscle glucose uptake.

AUTHOR CONTRIBUTIONS

J.P.K., A.C.B., J.L., and G.K.M. contributed to the study design; J.P.K., performed experiments; J.P.K. and A.C.B., performed laboratory analysis; J.P.K., A.C.B., and G.K.M interpreted findings; J.P.K. drafted the manuscript and all authors edited, revised, and approved final version of manuscript.

REFERENCES

1. **Balon TW, and Nadler JL**. Evidence that nitric oxide increases glucose transport in skeletal muscle. *Journal of applied physiology (Bethesda, Md : 1985)* 82: 359-363, 1997.

2. **Blair DR, Funai K, Schweitzer GG, and Cartee GD**. A myosin II ATPase inhibitor reduces force production, glucose transport, and phosphorylation of AMPK and TBC1D1 in electrically stimulated rat skeletal muscle. *Am J Physiol Endocrinol Metab* 296: E993-E1002, 2009.

3. **Bradley SJ, Kingwell BA, and McConell GK**. Nitric oxide synthase inhibition reduces leg glucose uptake but not blood flow during dynamic exercise in humans. *Diabetes* 48: 1815-1821, 1999.

4. **Brenman JE, Chao DS, Xia H, Aldape K, and Bredt DS**. Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell* 82: 743-752, 1995.

5. **Chambers MA, Moylan JS, Smith JD, Goodyear LJ, and Reid MB**. Stretch-stimulated glucose uptake in skeletal muscle is mediated by reactive oxygen species and p38 MAP-kinase. *J Physiol* 587: 3363-3373, 2009.

6. **Chang YS, Yaccino JA, Lakshminarayanan S, Frangos JA, and Tarbell JM**. Shear-induced increase in hydraulic conductivity in endothelial cells is mediated by a nitric oxide-dependent mechanism. *Arterioscler Thromb Vasc Biol* 20: 35-42, 2000.

7. **Etgen GJ, Jr., Fryburg DA, and Gibbs EM**. Nitric oxide stimulates skeletal muscle glucose transport through a calcium/contraction- and phosphatidylinositol-3-kinase-independent pathway. *Diabetes* 46: 1915-1919, 1997.

8. **Godfrey EW, and Schwarte RC**. Nitric oxide and cyclic GMP regulate early events in agrin signaling in skeletal muscle cells. *Exp Cell Res* 316: 1935-1945, 2010.

9. **Goldspink G, Scutt A, Martindale J, Jaenicke T, Turay L, and Gerlach GF**. Stretch and force generation induce rapid hypertrophy and myosin isoform gene switching in adult skeletal muscle. *Biochem Soc Trans* 19: 368-373, 1991.

10. **Heinonen I, Saltin B, Kemppainen J, Nuutila P, Knuuti J, Kalliokoski K, and Hellsten Y**. Effect of nitric oxide synthase inhibition on the exchange of glucose and fatty acids in human skeletal muscle. *Nutr Metab (Lond)* 10: 43, 2013.

11. **Heo J, and Campbell SL**. Mechanism of redox-mediated guanine nucleotide exchange on redox-active Rho GTPases. *J Biol Chem* 280: 31003-31010, 2005.

12. **Higaki Y, Hirshman MF, Fujii N, and Goodyear LJ**. Nitric oxide increases glucose uptake through a mechanism that is distinct from the insulin and contraction pathways in rat skeletal muscle. *Diabetes* 50: 241-247, 2001.

13. **Hong YH, Betik AC, Premilovac D, Dwyer RM, Keske MA, Rattigan S, and McConell GK**. No effect of NOS inhibition on skeletal muscle glucose uptake during in situ hindlimb contraction in healthy and diabetic Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol* 308: R862-871, 2015.

14. **Hong YH, Frugier T, Zhang X, Murphy RM, Lynch GS, Betik AC, Rattigan S, and McConell GK**. Glucose uptake during contraction in isolated skeletal muscles from neuronal nitric oxide synthase mu knockout mice. *Journal of applied physiology (Bethesda, Md : 1985)* 118: 1113-1121, 2015.

15. **Hong YH, Yang C, Betik AC, Lee-Young RS, and McConell GK**. Skeletal muscle glucose uptake during treadmill exercise in neuronal nitric oxide synthase-mu knockout mice. *Am J Physiol Endocrinol Metab* 310: E838-845, 2016.

16. **Hornberger TA, Stuppard R, Conley KE, Fedele MJ, Fiorotto ML, Chin ER, and Esser KA**. Mechanical stimuli regulate rapamycin-sensitive signalling by a phosphoinositide 3-kinase-, protein kinase B- and growth factor-independent mechanism. *Biochem J* 380: 795-804, 2004.

17. **Huang PL, Dawson TM, Bredt DS, Snyder SH, and Fishman MC**. Targeted disruption of the neuronal nitric oxide synthase gene. *Cell* 75: 1273-1286, 1993.

18. **Ihlemann J, Ploug T, Hellsten Y, and Galbo H**. Effect of tension on contraction-induced glucose transport in rat skeletal muscle. *Am J Physiol* 277: E208-214, 1999.

19. **Ito N, Ruegg UT, Kudo A, Miyagoe-Suzuki Y, and Takeda S**. Activation of calcium signaling through Trpv1 by nNOS and peroxynitrite as a key trigger of skeletal muscle hypertrophy. *Nat Med* 19: 101-106, 2013.

20. **Ito Y, Obara K, Ikeda R, Ishii M, Tanabe Y, Ishikawa T, and Nakayama K**. Passive stretching produces Akt- and MAPK-dependent augmentations of GLUT4 translocation and glucose uptake in skeletal muscles of mice. *Pflugers Arch* 451: 803-813, 2006.

21. **Iwata M, Hayakawa K, Murakami T, Naruse K, Kawakami K, Inoue-Miyazu M, Yuge L, and Suzuki S**. Uniaxial cyclic stretch-stimulated glucose transport is mediated by a ca-dependent mechanism in cultured skeletal muscle cells. *Pathobiology* 74: 159-168, 2007.

22. **JeBailey L, Rudich A, Huang X, Di Ciano-Oliveira C, Kapus A, and Klip A**. Skeletal muscle cells and adipocytes differ in their reliance on TC10 and Rac for insulin-induced actin remodeling. *Mol Endocrinol* 18: 359-372, 2004.

23. **Jensen TE, Sylow L, Rose AJ, Madsen AB, Angin Y, Maarbjerg SJ, and Richter EA**. Contraction-stimulated glucose transport in muscle is controlled

by AMPK and mechanical stress but not sarcoplasmatic reticulum Ca(2+) release. *Mol Metab* 3: 742-753, 2014.

24. **Kingwell BA, Formosa M, Muhlmann M, Bradley SJ, and McConell GK**. Nitric oxide synthase inhibition reduces glucose uptake during exercise in individuals with type 2 diabetes more than in control subjects. *Diabetes* 51: 2572-2580, 2002.

25. **Kleinert M, Parker BL, Fritzen AM, Knudsen JR, Jensen TE, Kjøbsted R, Sylow L, Ruegg M, James DE, and Richter EA**. Mammalian target of rapamycin complex 2 regulates muscle glucose uptake during exercise in mice. *The Journal of Physiology* 595: 4845-4855, 2017.

26. **Lau KS, Grange RW, Isotani E, Sarelius IH, Kamm KE, Huang PL, and Stull JT**. nNOS and eNOS modulate cGMP formation and vascular response in contracting fast-twitch skeletal muscle. *Physiol Genomics* 2: 21-27, 2000.

27. **Lee-Young RS, Griffee SR, Lynes SE, Bracy DP, Ayala JE, McGuinness OP, and Wasserman DH**. Skeletal muscle AMP-activated protein kinase is essential for the metabolic response to exercise in vivo. *J Biol Chem* 284: 23925-23934, 2009.

28. **Linden KC, Wadley GD, Garnham AP, and McConell GK**. Effect of l-arginine infusion on glucose disposal during exercise in humans. *Med Sci Sports Exerc* 43: 1626-1634, 2011.

29. **Martin IK, Katz A, and Wahren J**. Splanchnic and muscle metabolism during exercise in NIDDM patients. *Am J Physiol* 269: E583-590, 1995.

30. **Merry TL, Dywer RM, Bradley EA, Rattigan S, and McConell GK**. Local hindlimb antioxidant infusion does not affect muscle glucose uptake during in situ contractions in rat. *J Appl Physiol* 108: 1275-1283, 2010.

31. **Merry TL, Lynch GS, and McConell GK**. Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction. *Am J Physiol Regul Integr Comp Physiol* 299: R1656-1665, 2010.

32. **Merry TL, Steinberg GR, Lynch GS, and McConell GK**. Skeletal muscle glucose uptake during contraction is regulated by nitric oxide and ROS independently of AMPK. *Am J Physiol Endocrinol Metab* 298: E577-585, 2010.

33. **Palomero J, Pye D, Kabayo T, and Jackson MJ**. Effect of passive stretch on intracellular nitric oxide and superoxide activities in single skeletal muscle fibres: influence of ageing. *Free Radic Res* 46: 30-40, 2012.

34. **Pattwell DM, McArdle A, Morgan JE, Patridge TA, and Jackson MJ**. Release of reactive oxygen and nitrogen species from contracting skeletal muscle cells. *Free Radic Biol Med* 37: 1064-1072, 2004.

35. **Richter EA, and Hargreaves M**. Exercise, GLUT4, and skeletal muscle glucose uptake. *Physiol Rev* 93: 993-1017, 2013.

36. **Roberts CK, Barnard RJ, Jasman A, and Balon TW**. Acute exercise increases nitric oxide synthase activity in skeletal muscle. *Am J Physiol* 277: E390-394, 1999.

37. **Roberts CK, Barnard RJ, Scheck SH, and Balon TW**. Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am J Physiol* 273: E220-225, 1997.

38. **Ross RM, Wadley GD, Clark MG, Rattigan S, and McConell GK**. Local nitric oxide synthase inhibition reduces skeletal muscle glucose uptake but not capillary blood flow during in situ muscle contraction in rats. *Diabetes* 56: 2885-2892, 2007.

39. **Rottman JN, Bracy D, Malabanan C, Yue Z, Clanton J, and Wasserman DH**. Contrasting effects of exercise and NOS inhibition on tissue-specific fatty acid and glucose uptake in mice. *Am J Physiol Endocrinol Metab* 283: E116-123, 2002.

40. **Roy D, Perreault M, and Marette A**. Insulin stimulation of glucose uptake in skeletal muscles and adipose tissues in vivo is NO dependent. *Am J Physiol* 274: E692-699, 1998.

41. **Selvakumar B, Hess DT, Goldschmidt-Clermont PJ, and Stamler JS**. Co-regulation of constitutive nitric oxide synthases and NADPH oxidase by the small GTPase Rac. *FEBS Lett* 582: 2195-2202, 2008.

42. **Silveira LR, Pereira-Da-Silva L, Juel C, and Hellsten Y**. Formation of hydrogen peroxide and nitric oxide in rat skeletal muscle cells during contractions. *Free Radic Biol Med* 35: 455-464, 2003.

43. **Soltow QA, Lira VA, Betters JL, Long JH, Sellman JE, Zeanah EH, and Criswell DS**. Nitric oxide regulates stretch-induced proliferation in C2C12 myoblasts. *J Muscle Res Cell Motil* 31: 215-225, 2010.

44. **Sylow L, Jensen TE, Kleinert M, Mouatt JR, Maarbjerg SJ, Jeppesen J, Prats C, Chiu TT, Boguslavsky S, Klip A, Schjerling P, and Richter EA**. Rac1 is a novel regulator of contraction-stimulated glucose uptake in skeletal muscle. *Diabetes* 62: 1139-1151, 2013.

45. **Sylow L, Moller LL, Kleinert M, Richter EA, and Jensen TE**. Stretch-stimulated glucose transport in skeletal muscle is regulated by Rac1. *J Physiol* 593: 645-656, 2015.

46. **Sylow L, Moller LLV, Kleinert M, D'Hulst G, De Groote E, Schjerling P, Steinberg GR, Jensen TE, and Richter EA**. Rac1 and AMPK Account for the Majority of Muscle Glucose Uptake Stimulated by Ex Vivo Contraction but Not In Vivo Exercise. *Diabetes* 66: 1548-1559, 2017.

47. **Tatsumi R, Hattori A, Ikeuchi Y, Anderson JE, and Allen RE**. Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. *Mol Biol Cell* 13: 2909-2918, 2002.

48. **Tidball JG, Lavergne E, Lau KS, Spencer MJ, Stull JT, and Wehling M**. Mechanical loading regulates NOS expression and activity in developing and adult skeletal muscle. *Am J Physiol* 275: C260-266, 1998.

49. **Tidball JG, Spencer MJ, Wehling M, and Lavergne E**. Nitric-oxide synthase is a mechanical signal transducer that modulates talin and vinculin expression. *J Biol Chem* 274: 33155-33160, 1999.

50. **Vandenburgh H, and Kaufman S**. In vitro model for stretch-induced hypertrophy of skeletal muscle. *Science* 203: 265-268, 1979.

51. **Wang Z, Oh E, Clapp DW, Chernoff J, and Thurmond DC**. Inhibition or ablation of p21-activated kinase (PAK1) disrupts glucose homeostatic mechanisms in vivo. *J Biol Chem* 286: 41359-41367, 2011.

52. **Witczak CA, Jessen N, Warro DM, Toyoda T, Fujii N, Anderson ME, Hirshman MF, and Goodyear LJ**. CaMKII regulates contraction- but not insulin-induced glucose uptake in mouse skeletal muscle. *Am J Physiol Endocrinol Metab* 298: E1150-1160, 2010.

53. **Wojtaszewski JF, Higaki Y, Hirshman MF, Michael MD, Dufresne SD, Kahn CR, and Goodyear LJ**. Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice. *J Clin Invest* 104: 1257-1264, 1999.

54. **Wozniak AC, and Anderson JE**. The dynamics of the nitric oxide release-transient from stretched muscle cells. *Int J Biochem Cell Biol* 41: 625-631, 2009.

55. **Zhang JS, Kraus WE, and Truskey GA**. Stretch-induced nitric oxide modulates mechanical properties of skeletal muscle cells. *Am J Physiol Cell Physiol* 287: C292-299, 2004.

FIGURE LEGENDS

Figure 1. NOS inhibition does not attenuate stretch-stimulated skeletal muscle glucose uptake. Stretch-stimulated 2-deoxyglucose uptake in EDL muscles from C57BL/6 mice incubated for 30 min with or without the NOS inhibitors L-NMMA (100 μM) or L-NAME (100 μM) (n = 4-10 per group). Data are means ± SEM. *** P < 0.001 vs. Basal.

Figure 2. Deletion of eNOS or nNOSμ does not affect stretch-stimulated skeletal muscle glucose uptake. 2-deoxyglucose uptake at rest (basal) and during stretch in EDL muscles of A) C57BL/6 and $eNOS^{-/-}$ mice (n = 5-9 per group) and B) $nNOS\mu^{+/+}$ and $nNOS\mu^{-/-}$ mice (n = 6-7 per group). Data are means ± SEM. *** P < 0.001 compared to basal. **§** P < 0.05 main effect for genotype.

Figure 3. Passive stretch increases phosphorylation of skeletal muscle PAK1/2 and p38 MAPK independently of NOS activation. A) NOS activity of EDL muscles at rest (basal) or following passive stretch (n = 4 per group). Immunoblot quantifications for B) p-$PAK1/2^{Thr423/402}$, C) p-p38 $MAPK^{Thr180/Tyr182}$, D) p-$AMPK^{Thr172}$, E) p-$CaMKII^{Thr286}$, and E) representative immunoblots of EDL muscles at rest (basal) or following passive stretch (n = 4 per group). Data are means ± S.E.M.* P < 0.05 vs. Basal.

FIGURE 1

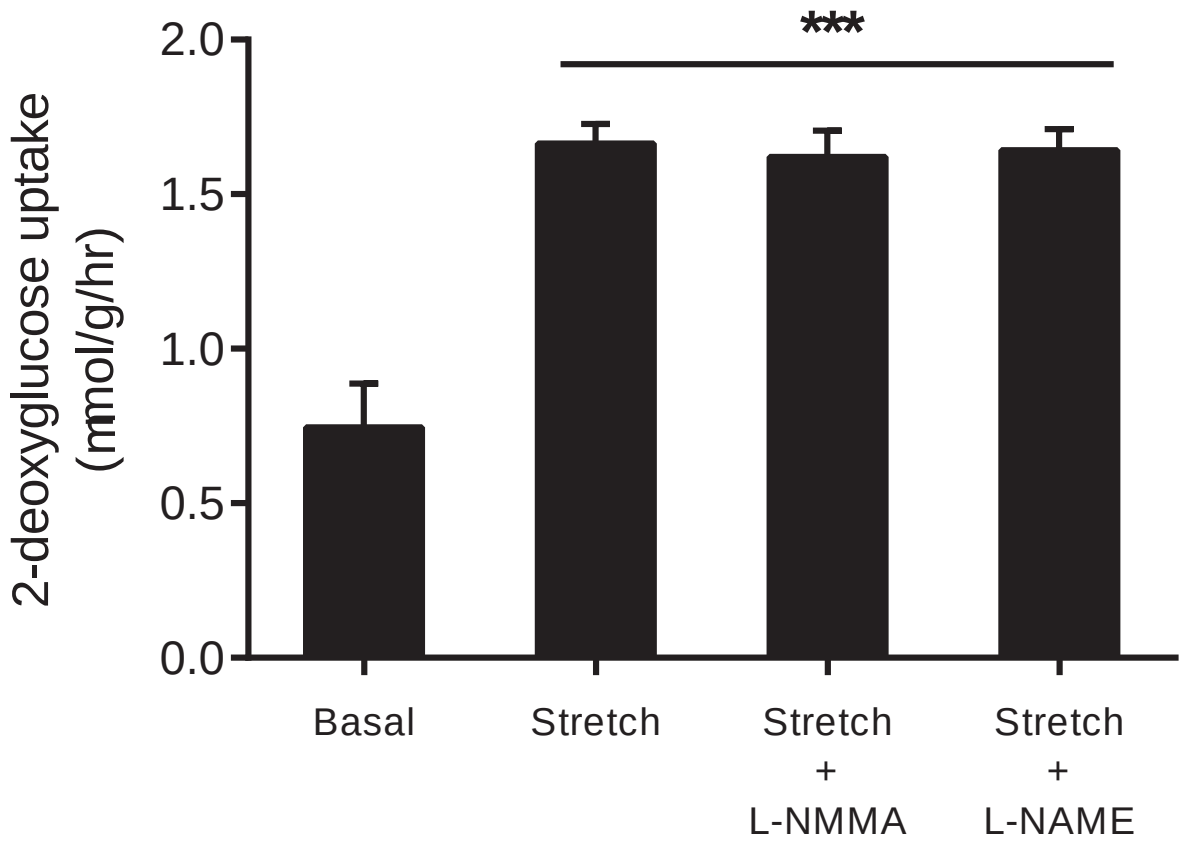

***
2.0
1.5
1.0
0.5
0.0
2-deoxyglucose uptake
(mmol/g/hr)
Basal
Stretch
Stretch
+
L-NMMA
Stretch
+
L-NAME

FIGURE 2

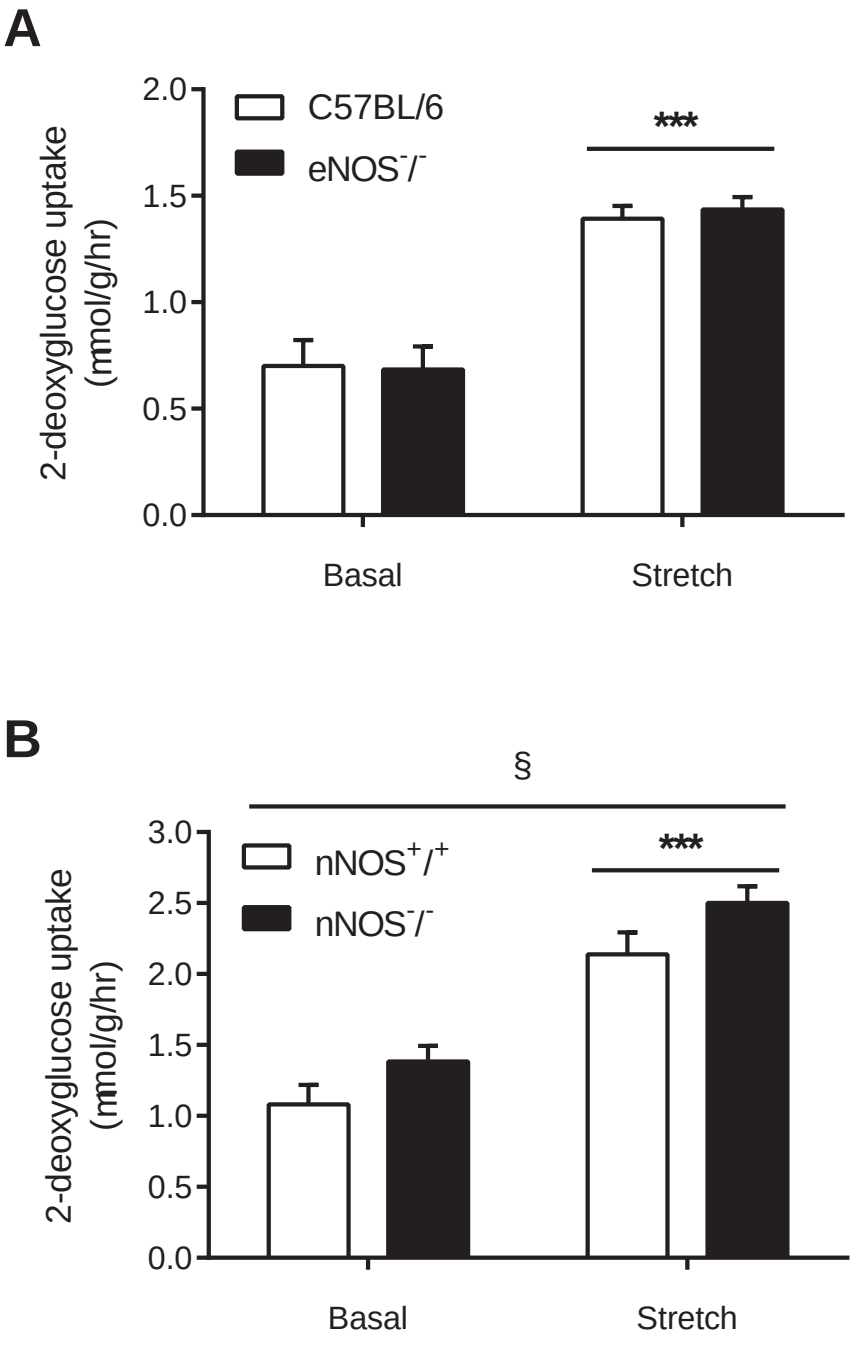
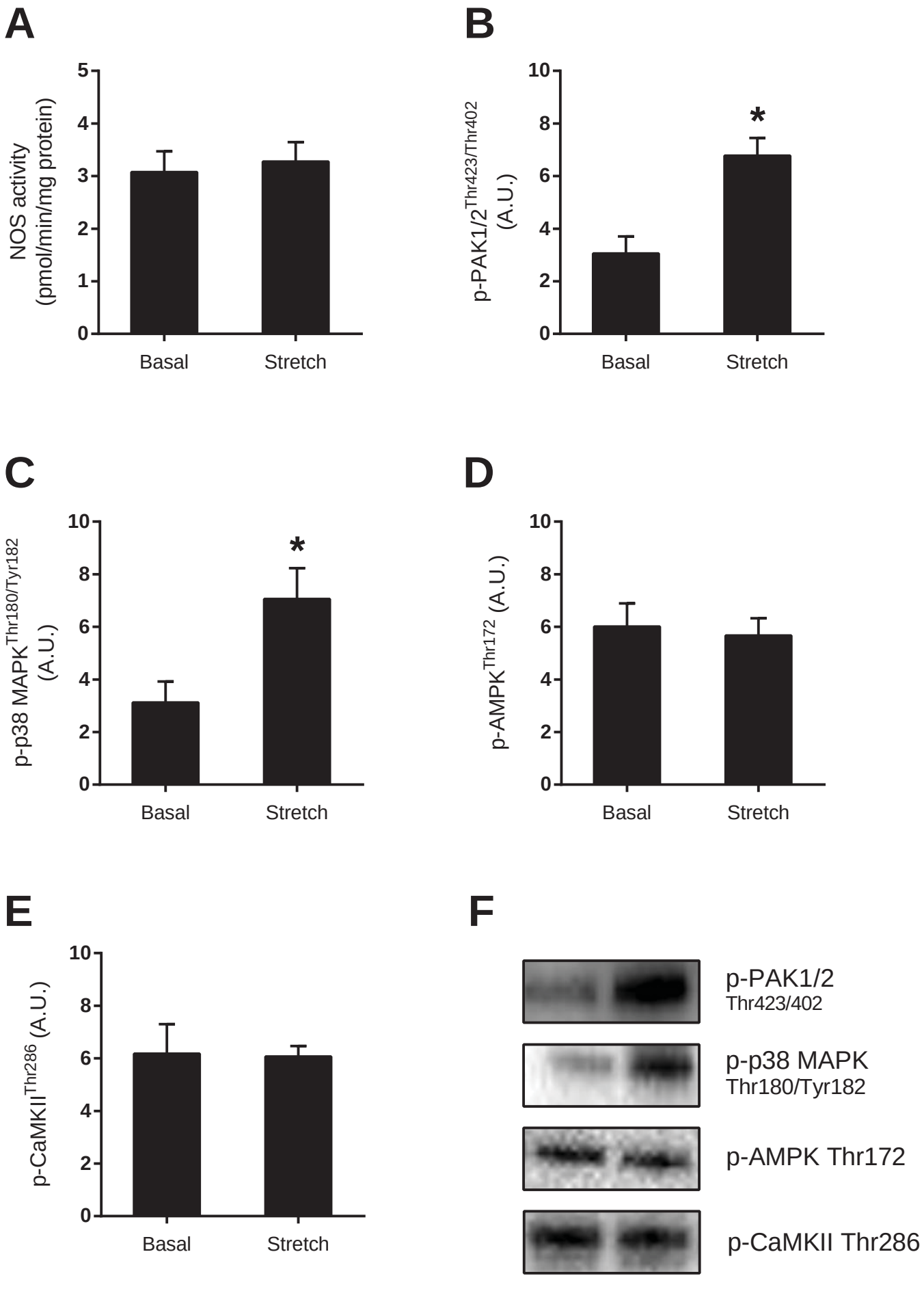

A
2.0
1.5
1.0
0.5
0.0
2-deoxyglucose uptake (mmol/g/hr)
C57BL/6
eNOS-/-
***
Basal
Stretch
B
§
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2-deoxyglucose uptake (mmol/g/hr)
nNOS+/+
nNOS-/-
***
Basal
Stretch

FIGURE 3


A
NOS activity (pmol/min/mg protein)
5
4
3
2
1
0
Basal
Stretch
B
p-PAK1/2Thr423/Thr402 (A.U.)
10
8
6
4
2
0
*
Basal
Stretch
C
p-p38 MAPKThr180/Tyr182 (A.U.)
10
8
6
4
2
0
*
Basal
Stretch
D
p-AMPKThr172 (A.U.)
10
8
6
4
2
0
Basal
Stretch
E
p-CaMKIIThr286 (A.U.)
10
8
6
4
2
0
Basal
Stretch
F
p-PAK1/2
Thr423/402
p-p38 MAPK
Thr180/Tyr182
p-AMPK Thr172
p-CaMKII Thr286